THE ORIGIN OF CIGUATERA IN PLATYPUS BAY, AUSTRALIA

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Platypus Bay on the northwestern side of Fraser Island is the only site in Queensland known to frequently harbour ciguateric fishes. Platypus Bay is not typical of areas normally associated with ciguateric fishes as it contains no corals but has a sandy bottom covered with an unattached green macroalgae (*Cladophora* sp.). Benthic biodetritus samples sieved from the *Cladophora* during seven sampling trips between May 1988 and February 1990 contained *Gambierdiscus toxicus* with mean population densities of 4–556 cells per gram of *Cladophora*. Biodetritus samples from six of the trips were extracted for toxins. Putative major and minor gambiertoxins (precursors of the ciguatoxins) were detected, suggesting that these *G. toxicus* populations are the origin of the toxins in ciguateric fishes caught in Platypus Bay. However, gambiertoxins were detected from only one of the six samples. This indicates that not all strains of *G. toxicus* produce these toxins in the wild. The concentrations of major and minor gambiertoxins produced by these wild *G. toxicus* can be considerably greater than the highest levels found from cultured *G. toxicus* clones isolated from Platypus Bay. 'Super-producing' strains of *G. toxicus* are hypothesised to explain the high concentrations of these toxins.

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Ciguatera is a disease caused by eating fish contaminated with ciguatoxins which are thought to originate from less-oxidised precursors called gambiertoxins that are produced by the benthic dinoflagellate *Gambierdiscus toxicus*. Gambiertoxins are produced by only a minority of cultured *G. toxicus* strains (Holmes et al., 1991). In Australia, *G. toxicus* has been found along the east coast from Flat Rock, E of Brisbane, to Alexandra Reef N of Cairns, co-occurring with corals (Gillespie et al., 1985a; Gillespie, 1987). *G. toxicus* is the most common benthic dinoflagellate on Queensland coral reefs, except on some reef flats where *Ostreopsis* spp. dominates (Gillespie et al., 1985a). Population densities of *G. toxicus* on Queensland reefs are generally <5 cells/g of substrate. Flinders Reef in south Queensland is an exception which seasonally produces >1,800 *G. toxicus*/g of macroalgal substrate (Gillespie et al., 1985a). However, gambiertoxins and ciguatoxins have not been extracted from *G. toxicus* or fish from Flinders Reef (Gillespie et al.,1985b; Lewis et al.,1988a).

Platypus Bay, on the northwestern side of Fraser Island (Fig.1), is the only site in Queensland known to frequently harbour ciguateric fishes (Lewis & Endean,1983,1984; Gillespie et al., 1986; Lewis,1987). Mackerels (*Scomberomorus* spp.), especially narrow-barred Spanish mackerel (*S. commersoni*) caught near the mouth of Wathumba Creek, have caused most of these poisonings (Lewis & Endean,1983; Gillespie et al.,1986; Lewis,1987; Lewis et al., 1988b). Since prohibition, in 1987, on the capture of *S. commersoni* and barracuda (*Sphyraena jello* and *Agrioposphyraena* spp.) from Platypus Bay, the majority of cases of ciguatera in Queensland have been caused by demersal fish from the Great Barrier Reef (unpubl. data). However, small demersal fishes from Platypus Bay have caused ciguatera, including the blotched javelin (*Pomadasys maculatus*) (Lewis et al.,1988b) and less often, the rabbitfish *Siganus spinus* (unpubl. data). Ciguatera is typically associated with fishes of coral reefs (Randall,1958; Bagnis et al., 1989) so its occurrence in Platypus Bay where corals are absent seems unusual. The benthic biodetritus of Platypus Bay was examined for dinoflagellates and extracted for toxins. Putative gambiertoxins from a biodetrital fraction with large numbers of *G. toxicus* indicates that the origin of ciguatoxins in fishes caught in Platypus Bay is likely to be *G. toxicus* in Platypus Bay.

MATERIALS AND METHODS

Biodetritus from Platypus Bay

Large areas of the bottom of Platypus Bay

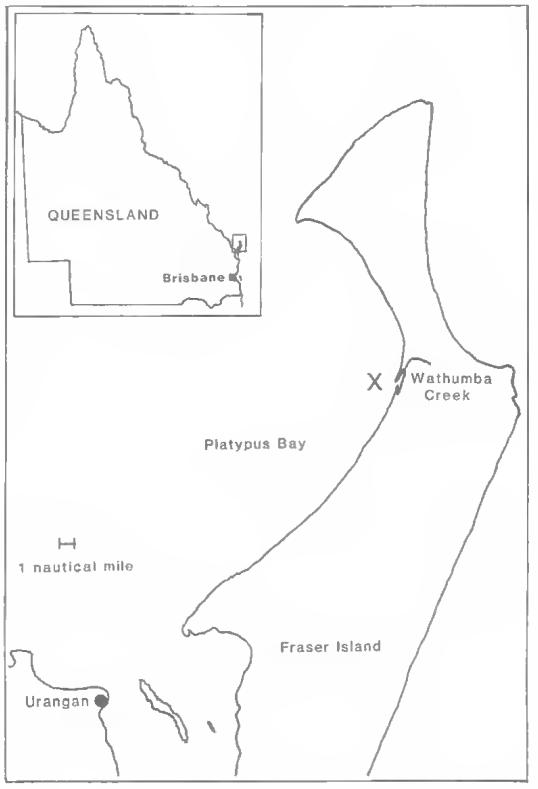

FIG. 1. Map of Platypus Bay, Fraser Island. *Cladophora* samples were collected by scuba divers from the site marked with a cross.

(Fig.1) are perennially covered with several centimetres of a non-attached green macroalgae (*Cladophora* sp.). Samples of *Cladophora* were collected by scuba divers during seven sampling trips between April 1988 and February 1990 from about 1.5 nautical miles E of the mouth of Wathumba Creek in c.15 m of water (Fig.1). Samples were collected in Bitran zip-lock plastic bags, transported to Brisbane, stored at 4°C overnight and then processed. Insufficient material was collected during the July 1988 trip for toxicity studies but this sample was examined for benthic dinoflagellates. The contents of the plastic bags from the remaining six trips were shaken and poured onto 2mm, 500μm, 250μm and 45μm diameter plankton mesh sieves. The filtrate from the 45μm sieve was vacuum filtered on Whatman Number 1 filter paper; the filtrate obtained after this was discarded. The fractions retained by the 500μm, 250μm and 45μm diameter sieves and the filter papers were extracted for toxins. A 590g sample of *Cladophora* (retained on the 2mm sieve) collected in May 1988 was also homogenised and extracted for toxins. Benthic dinoflagellates were identified with a light microscope (Fukuyo,1981). Cell numbers were calculated from counts of 1ml subsamples of sieved plastic bag samples of *Cladophora* (n=2–4 plastic bag samples) using a Sedgewick-Rafter counting chamber. Scanning electron micrographs of formalin (5%) fixed biodetrital fractions were prepared (Holmes et al., 1990). *Coolia monotis* was identified by SEM examination of cultured cells isolated from a *Cladophora* sample collected in July 1988.

Solvent Extraction of Sieved Fractions

Fractions were homogenised for 20 minutes in acetone (3-times, 3:1, v:v, acetone:sample) using a Ystral air powered homogeniser. The 45-250μm fractions were additionally homogenised with methanol (1-time, 3:1, v:v, methanol: sample). The extracts for each size fraction were pooled, vacuum filtered through Whatman GF/A glass fibre filters and dried under vacuum. The dried extracts were resuspended in 9:1 methanol-water and then separated into hexane-, diethyl ether-, butanol- and water-soluble fractions (Holmes et al.,1990). Gambiertoxins (or ciguatoxins) extracted from these samples would partition into the diethyl ether fractions, whereas maitotoxins partition into both the diethyl ether and butanol fractions (Holmes et al.,1990,1991; Holmes & Lewis,1992). Diethyl ether fractions which induced gambiertoxin-like bioassay signs in mice were further characterised after separation by silicic acid column chromatography (Holmes et al.,1990; Holmes & Lewis,1992).

Mouse Bioassay

Fractions were dried under vacuum and finally freed of solvent under a stream of N_2, resuspended in 0.5 ml of 1% or 5% Tween 60 saline and injected intraperitoneally (i.p.) into 18–21g Quackenbush strain mice (either sex) at a maximum dose of 1g of dried fraction weight per kg mouse body weight. One mouse was injected per dose, with 2 or 3 mice injected per fraction. Where appropriate, 10% and 90% of fractions were injected (for example, where 10% was not lethal and 90% was ≤ maximum injectable dose). Mice were observed intermittently over 24 hours and signs and death-times recorded. Fractions where considered non-toxic if injection of a maximal dose was not lethal. Lethal fractions were characterised on the basis of the signs and death-times displayed by mice compared with the responses induced by authentic gambiertoxins,

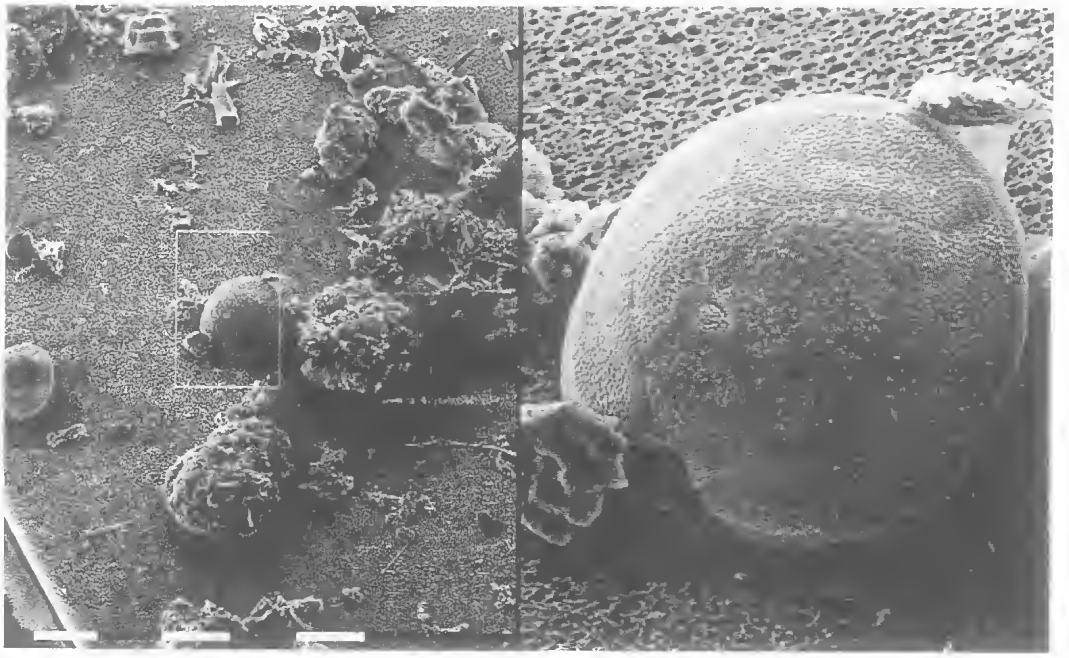

FIG. 2. Scanning electron micrograph of sieved Platypus Bay biodetritus (45-250μm) containing *Gambierdiscus toxicus*. A *G. toxicus* is shown enclosed in the box. Scale bar = 50μm.

ciguatoxins and maitotoxins. Gambiertoxins were quantified using the dose *vs* death-time equation for the major gambiertoxin; log (dose)= 3.2 log $(1+t^{-1})$, where dose is in mouse units (MU) and t = death-time in hours (Holmes & Lewis, 1992). One MU is defined as an i.p. LD_{50} dose.

Gambiertoxins, ciguatoxins and maitotoxins are all slow acting (Holmes et al.,1990; Lewis et al.,1991; Holmes & Lewis,1992). For each, i.p. injection of a dose one-tenth of that which would kill a mouse in 30 minutes, would also be lethal. Fast-acting toxins were therefore defined as fractions which killed mice in 30 minutes (or less) but which were not lethal at one-tenth of this dose. The limit of detection of gambiertoxins from non-toxic fractions was calculated from the maximum amount of material that could be injected into mice which was non-toxic, assuming 0.5 MU of gambiertoxin can be detected from the bioassay signs displayed by a mouse.

RESULTS

Platypus Bay biodetritus contains (Fig.2) cells of *G. toxicus* as described by Fukuyo (1981). *G. toxicus* was found in sieved fractions collected from all seven sampling trips with mean population densities of 4–556 cells/g of *Cladophora* (Fig.3). There was considerable variation in the size of the *G. toxicus* populations, with the May 1988 sample having the highest cell densities and May 1989 the second lowest. There was no obvious relationship between *G. toxicus* population

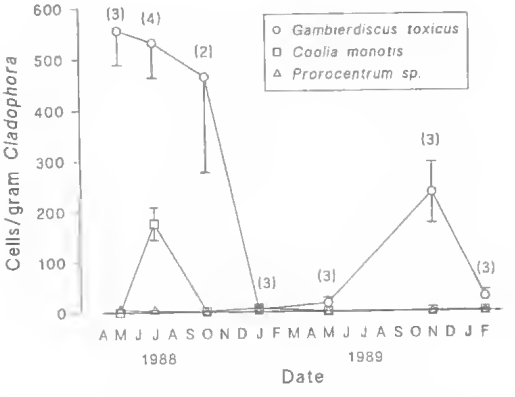

FIG. 3. Population densities of *G. toxicus*, *Coolia monotis* and *Prorocentrum* sp. on *Cladophora* from Platypus Bay. Samples collected May 1988 to February 1990. Shown are means ±1 standard error, 2 to 4 replicate samples as indicated by numbers in parenthesis. Sieved fractions were tested for toxicity for all samples except the July 1988 sample.

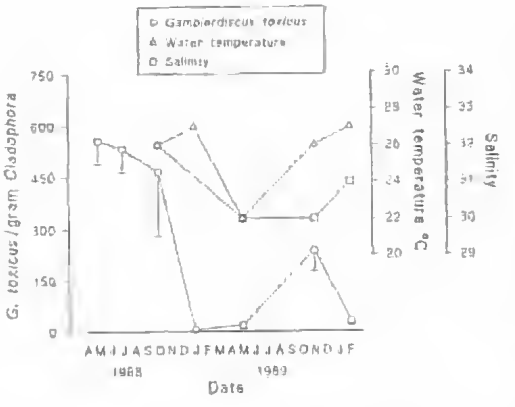

FIG. 4. Population densities of *Gambierdiscus toxicus* and Platypus Bay sea water temperatures and salinities. Samples were collected between May 1988 and February 1990. *G. toxicus* populations densities indicated are means -1 standard error.

densities and seawater temperature or salinity (Fig. 4).

Benthic dinoflagellates *C. monotis* and *Prorocentrum* sp. were also observed in biodetrital fractions but with generally smaller population densities than *G. toxicus* (Fig.3). A bloom of *C. monotis* (176 cells/g of *Cladophora*) was observed in July 1988 but this was less than half the *G. toxicus* cell density in the sample.

The yields and toxicity to mice of hexane-, diethyl ether-, butanol- and water-soluble fractions extracted from Platypus Bay biodetritus (Table 1) show that 28 (of 96) fractions were lethal to mice. Half of the lethal fractions were extracted from the 45–250μm sized biodetritus samples which encompasses the size range of *G. toxicus*. The May and October 1988 diethyl ether extracts of the 45–250μm sized biodetrital samples induced signs in mice that appeared as a composite of gambiertoxin and maitotoxin effects (Table 1). The diethyl ether extracts from the remaining four 45–250μm biodetrital fractions (Table 1) were non-toxic or contained a few MU of a fast-acting toxin(s). These four fractions would each have contained no more than 5–6 MU (assuming a limit of detection of 0.5 MU for the mouse bioassay) and were not characterised further. No other extracts induced gambiertoxin-like signs in mice.

TABLE 1. Yield and toxicity of extracts of sieved *Cladophora* sp. samples from Platypus Bay.

		9-5-88[a] (9.2kg)[b]		5-10-88 (8.5kg)		24-1-89 (29.0kg)		23-5-89 (13.6kg)		8-11-89 (8.1kg)		28-2-90 (8.6kg)	
Fraction size (mm)	Extract	Wt[c] (mg)	Toxin[d]	Wt (mg)	Toxin	Wt (mg)	Toxin	Wt (mg)	Toxin	Wt (mg)	Toxin	Wt (mg)	Toxin
2.0-0.5	Hexane	60		186		110		91		53		57	
	Diethyl ether	85	?	33	?/F	15	MTX	24		10		10	
	Butanol	188		200		60		56		60		57	
	Water	909		7		21		18		t3		3	
0.5-0.25	Hexane	123		13		499		162		29		65	
	Diethyl ether	47	?	3		1502		47		19		8	
	Butanol	110	MTX	3	MTX	55		54		138		25	
	Water	27	MTX	26		303		29		1043		9	
0.25-0.045	Hexane	1641		370		212		457		124	MTX	262	
	Diethyl ether	864	G/MTX	391	G/MTX	77	?/F	233		29	MTX/F	23	?/F
	Butanol	1355	MTX	48	MTX	64	MTX	74	MTX	70	MTX	122	MTX
	Water	36726		173		20		33	?	26	MTX	19	
Filter paper	Hexane	513		466		721		204		356		424	
	Diethyl ether	96		273	?	2038		583		59	?/F	65	MTX
	Butanol	168		90	?	85	MTX	52	MTX	226	MTX	329	
	Water	98		291		401		25		32		24	

[a] Date of collection
[b] Weight of *Cladophora* sp. sampled (shaken, sieved and hand squeezed free of water).
[c] Fraction weight.
[d] Toxicity (i.p.) to 18–21g mice (n = 2-3 per fraction); blank, indicates the fraction was not lethal up to a maximum dose of 1g fraction weight per kg mouse body weight;
MTX, indicates a lethal fraction that induced maitotoxin-like signs;
G/MTX, indicates a lethal fraction that induced a composite of gambiertoxin-like signs and MTX signs;
?, indicates a lethal fraction that induced neither ciguatoxin, gambiertoxin or maitotoxin-like signs;
F, indicates fast-acting toxin.

Silicic acid column chromatography of the May 1988 diethyl ether extract of the 45–250μm sized sample revealed that the 97:3 and 9:1 chloroform-methanol fractions were lethal to mice and induced signs identical to those from gambiertoxins (including hind-limb paralysis). Quantification of the 97:3 chloroform-methanol eluent using the dose vs death-time equation for the major gambiertoxin indicated 65±18 MU of toxin (mean±standard error, n=3), equivalent to 1.3×10^{-5} MU/cell of *G. toxicus*. Approximately 2 MU of toxin was recovered from the 9:1 chloroform-methanol eluent (3.9×10^{-7} MU/cell of *G. toxicus*). On the basis of mouse bioassay signs and column chromatography, the toxins in the 97:3 and 9:1 chloroform-methanol eluents were interpreted to be major and minor gambiertoxins, respectively. The chloroform eluent from the silicic acid column was non-toxic and the methanol eluent contained a fast-acting toxin that induced maitotoxin-like signs in mice. Gambiertoxins were not detected in eluents from a silicic acid column of the diethyl ether extract from the 45–250μm biodetritus sample collected in October 1988. The chloroform, 97:3 and 9:1 chloroform-methanol eluents of this sample were non-toxic to mice, the methanol eluent contained a maitotoxin-like toxin. The limit for detection of gambiertoxins using the mouse bioassay was 2-3 MU of toxin for these 97:3 and 9:1 chloroform-methanol eluents.

Only one hexane- and three water-soluble fractions were lethal to mice. Seventeen of the 28 lethal fractions (Table 1) induced maitotoxin-like signs in mice including all the butanol-soluble extracts from the 45–250μm sized biodetrital samples. Fast-acting toxins were detected in five of the diethyl ether-soluble extracts. Nine of the 28 lethal fractions (including four containing fast-acting toxins) produced signs in mice that were not consistent with those produced by either ciguatoxins, gambiertoxins or maitotoxins. The hexane-, diethyl ether- and butanol-soluble fractions extracted from the sample of *Cladophora* were non-toxic, the water-soluble fraction was lethal and induced maitotoxin-like signs in mice.

DISCUSSION

Extraction of putative major and minor gambiertoxins from a Platypus Bay biodetritus fraction containing large numbers of *G. toxicus* indicates that the origin of the ciguatoxins in Platypus Bay fishes is likely to be the *G. toxicus* in Platypus Bay. The extraction of similar gambiertoxins from cultures of a *G. toxicus* clone isolated from Platypus Bay (Holmes et al.,1991; Holmes & Lewis,1992) supports this hypothesis. Presumably, the gambiertoxins are biotransformed to ciguatoxins and accumulated in Platypus Bay fishes. Lewis & Sellin (1992) showed that the two known structural types of ciguatoxin are accumulated in the flesh of two species of ciguateric fish caught in Platypus Bay. These results indicate that the toxins that cause ciguatera do not originate only from coral reefs, but can be produced wherever substrates exist for *G. toxicus* and environmental conditions are suitable for the growth of gambiertoxin- producing strains of this dinoflagellate. Gambiertoxins have now been detected from wild *G. toxicus* harvested from the rhodophyte, *Jania* sp. (Murata et al.,1990) and the chlorophytes *Halimeda* sp. (Holmes et al.,1991) and *Cladophora* (present study). This indicates that gambiertoxin-production by *G. toxicus* is not dependent upon a particular class or species of macroalgal substrate.

The yield of the major gambiertoxin (1.3×10^{-5} MU/cell of *G. toxicus*) was similar to the yield from wild cells from Kiribati (Holmes et al., 1991), and greater than that extracted from cultured cells (Holmes et al.,1991; Holmes & Lewis, 1992). The yield of the minor gambiertoxin from Platypus Bay wild cells (3.9×10^{-7} MU/cell) was also an order of magnitude greater than from cultures of *G. toxicus* (Holmes & Lewis,1992). Consequently, the ratio of major to minor gambiertoxin (MU:MU) was similar for wild and cultured *G. toxicus* (1:0.03 and 1:0.04, respectively). However, only the major gambiertoxin was detected from wild cells from Kiribati indicating that the ratio of major:minor gambiertoxin is not constant in all strains of *G. toxicus* that produce gambiertoxins. The production of different relative quantities of the two gambiertoxins by *G. toxicus* may partly explain the differences in relative amounts of CTX-1, -2 and -3 that Lewis & Sellin (1992) found in the flesh of ciguateric fishes caught from Platypus Bay. Sufficient *G. toxicus* were extracted in the October 1988 and November 1989 biodetrital samples to have detected gambiertoxins if the concentrations of the major gambiertoxin per cell had been similar to the levels in the May 1988 sample. Holmes et al. (1991) showed that not all strains of *G. toxicus* produce gambiertoxins in culture. Our study shows that differences in toxin production between strains is not restricted to cultured *G. toxicus*.

It appears that some wild cells must be capable

of producing considerably greater amounts of gambiertoxins than the mean cell toxicity (10^{-5} MU/cell) of the toxic wild sample found in this study, given that the wild sample would likely contain a mixture of high and low gambiertoxin-producing strains. The high gambiertoxin-producing strains of *G. toxicus* (here after referred to as 'super-producing' strains) may increase the potential for ciguatera. Production of gambiertoxins in these super-producers may be partly controlled by environmental parameters. Holmes & Lewis (1992) showed that the concentrations (or type) of gambiertoxins produced by cultured clones of *G. toxicus* can change during the time they are maintained in culture. Therefore, the size of *G. toxicus* populations does not necessarily reflect their potential to cause ciguatera. The absence of ciguatera at Flinders Reef in southern Queensland, which seasonally harbours more than 1,800 *G. toxicus*/g of macroalgal substrate (Gillespie et al.,1985a,b; Lewis et al.,1988a) could therefore be explained by an absence of gambiertoxin (or super-gambiertoxin) producing strains of *G. toxicus* .

A number of *Prorocentrum* species produce toxins (Yasumoto et al.,1980; Nakajima et al., 1981; Dickey *et al.*,1990) while *C. monotis* is thought to be non-toxic (Yasumoto et al.,1980; Nakajima et al.,1981; Tindall et al.,1984). *Prorocentrum* and *C. monotis* are unlikely to have contributed significantly to the toxicity of Platypus Bay biodetrital fractions tested because of the small numbers of cells (<9cells/g *Cladophora*) present with *G. toxicus*. Additionally, there is no evidence that toxins produced by these species accumulate in fish to cause human illness. Approximately 30% of biodetrital fractions were lethal to mice and about 60% of these fractions induced maitotoxin-like signs, including all butanol-soluble extracts of the 45–250μm fractions. All of the 45–250μm fractions contained *G. toxicus* and therefore butanol-soluble extracts of these fractions would likely contain maitotoxins. Toxins which induce maitotoxin-like signs in mice have been extracted from the viscera of fishes (Yasumoto et al.,1976; Lewis et al.,1988a; Lewis et al.,1991), but there is no evidence for the bioaccumulation of maitotoxin or maitotoxin-like toxins in the flesh of Queensland fishes causing human illness. Mouse bioassay signs alone are unlikely to be diagnostic for maitotoxins as the benthic dinoflagellate *Ostreopsis siamensis* also produces a toxin (more-polar than maitotoxin) that induces similar signs in mice (Holmes et al.,1988). Fast-acting ciguatoxins have been reported from the flesh of ciguateric fishes from the Caribbean (Vernoux & Talha,1989) but authentic ciguatoxins, gambiertoxins and maitotoxins from Pacific Ocean sources have all proved to be slow-acting toxins in mice (Holmes et al.,1990; Holmes & Lewis,1992; Lewis et al.,1991). The dose vs death-time relationship in mice is therefore a useful method for differentiating toxins that produce otherwise similar bioassay signs in mice.

The *G. toxicus* populations in Platypus Bay are the second highest reported from Queensland (Gillespie et al.,1985a). However, these populations are smaller than the 4.5 x 10^5cells/g of macroalgae reported from the Gambier Islands (Bagnis et al.,1985). Bagnis et al. (1990) found 100-fold increases in *G. toxicus* populations can occur in less than two weeks. The environmental parameters which control the size of *G. toxicus* populations and the proportion of these cells which produce gambiertoxins (and possibly super-producers) need to be determined before the potential risk of ciguatera can be predicted from *G. toxicus* populations.

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